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Occupational exposure to organic solvents and K-*ras* mutations in exocrine pancreatic cancer

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Occupational exposure to hydrocarbon solvents has been found to be associated with an increased risk of exocrine pancreatic cancer (EPC), the human tumor with the highest prevalence of K-*ras* mutations. *Ras* genes are critical DNA targets for chemical carcinogens. We analysed the relationship between past occupational exposure to hydrocarbon solvents and mutations in codon 12 of the K-*ras* gene in 107 incident cases of EPC. Information on occupational factors and life-style was obtained from personal interviews conducted during hospital stay. Occupational exposure to hydrocarbon solvents (aliphatic, aromatic, chlorinated, benzene, other organic solvents) was examined using two methods: expert assessment and the Finnish job-exposure matrix (Finjem). Exposure among K-*ras* mutated EPC cases ($n = 83$) was compared with that of K-*ras* wild-type EPC cases ($n = 24$). An association between K-*ras* mutations and solvent exposure was observed with Finjem but barely so with the expert assessment. Over 7-fold increased odds ratios (OR) were found for every group of solvents evaluated with Finjem (all $P < 0.05$). On the basis of the expert assessment, K-*ras* mutations were significantly associated only with exposure to benzene in men (OR = 7.07, $P < 0.05$). When requiring exposure to have occurred according to both the experts and Finjem, over 4-fold risks were obtained for aromatic, aliphatic, and for 'any hydrocarbon solvent'. A significantly higher proportion of cases with a mutation from glycine to valine (GGT⊖GTT) or to aspartic acid (GGT⊖GAT) were exposed to a hydrocarbon solvent. The results raise the possibility that hydrocarbon solvents might be involved in the pathogenesis of EPC, possibly through indirect modulation of K-*ras* activation. Since this is only the first study on occupational exposures and K-*ras* mutations in EPC, studies able to refute or to confirm the findings are required before public health implications, if any, are assessed.

Introduction

While the high lethality of pancreatic cancer makes it a leading cause of cancer mortality in Western countries, knowledge on its etiology is limited (1,2). The main risk factors identified include the advance of age, male sex, cigarette smoking and possibly diabetes. A recent meta-analysis suggests that the risk of pancreatic cancer may also be increased by occupational factors, such as exposure to organic solvents (2).

Mutations in *ras* genes are the most frequent oncogenic alteration in human cancer, and a major example of activation by point mutation (3). Exocrine pancreas cancer (EPC) is the human tumor with the highest prevalence at diagnosis of K-*ras* mutations (ranging from 75–85%) (1,3–5). Molecular pathology and epidemiologic studies suggest that wild-type K-*ras* carcinomas of the pancreas may arise through a genetic pathway distinct from carcinomas that harbour a K-*ras* mutation (1,4,5). In EPC, all *ras* mutations are limited to the K-*ras* gene, they are an early event, and practically all occur in codon 12. Whether their occurrence and persistence in human neoplasias is associated with clinical, environmental or other factors is yet unknown (1). The relation between tobacco and alcohol consumption, and K-*ras* activation is unclear in EPC (4,6). Coffee (4,7) and some organochlorine compounds (1,5) have been tentatively associated with the prevalence of K-*ras* mutations at diagnosis in EPC.

Some agents present in the occupational environment may induce or promote neoplasms with *ras* alterations in animal models (8–11). In humans, differing results have risen from studies assessing a potential link between occupational exposures, including organic solvents (12–17), and *ras* alterations, both among healthy workers (13–19) and cancer patients (12,20–27), but no evidence is yet available for EPC.

The workplace represents the major source of exposure to organic solvents. Hydrocarbon solvents are present in the chemical, engineering, construction and manufacturing industries, among others. Exposure to these compounds is a well-known cancer hazard (28). The International Agency for Research on Cancer has classified the evidence of carcinogenicity to humans as 'sufficient' for benzene (29), 'limited' for several aliphatic solvents (30), and 'limited' or 'inadequate' for some other aromatic solvents (29,31,32).

The objective of the present study was to analyse the relationship between past occupational exposure to organic solvents and mutations in the K-*ras* gene in patients with EPC.

Subjects and methods

Methods have been previously described (1,4,33–35). Briefly, subject recruitment took place in 1992–1995 at five general hospitals in eastern Spain,

Abbreviations: EPC, exocrine pancreatic cancer; Finjem, Finnish Job-Exposure Matrix; IH, industrial hygienists; JEMs, job-exposure matrixes; OR, odds ratio; CI, confidence interval.

where 185 incident cases of EPC were prospectively identified. All their discharge diagnoses were reviewed by a panel of experts blinded to molecular results (35). All cases were also independently reviewed by the study reference pathologists, unaware of the original diagnoses.

The present report is based on 107 EPC patients with known K-*ras* status and occupational data. There were no significant differences between them and the remaining EPC cases with respect to sex, education, study site, tumor stage, duration of the interview, and consumption of coffee, tobacco and alcohol, except that the included subjects were slightly younger (1,4). Of the 107 cases, 83 (77.6%) harboured a K-*ras* mutation and 24 did not. Their mean age was 64.2 years (SD 12.2, range 36.8–88.6), and 42% were female. There were no differences in the frequency of mutations according to age, sex, education and tumor stage at diagnosis (4). Wild-type cases had smoked slightly more and drunk significantly less coffee than mutated cases (4). The study protocol was approved by the Ethics Committee of the participating hospitals, and patients gave informed consent to be included in the study.

Detection of K-ras mutations

Paraffin-embedded tumor samples were used for the molecular analyses. Methods to detect mutations have been described in detail (1,4,6). Briefly, DNA was extracted and amplified in two steps by nested PCR; in the second amplification reaction, an artificial BstNI restriction endonuclease site was introduced to discriminate between wild-type and mutated K-*ras* codon 12 sequences. Products were analysed by acrylamide gel electrophoresis and ethidium bromide staining. To characterize the nucleotide substitution in codon 12, all mutated samples were further analysed using a similar RFLP-based approach, as described elsewhere (6). DNA from oral mucosal scrapings was used as normal control and DNA from pancreas cancer cell lines or tumours were used as controls for the Val, Asp, Arg, Cys and Ser mutations. Interpretation of digestion products electrophoresis was performed independently by two investigators (NM and FXR). When discordant results were obtained, the analysis was repeated and results evaluated again. This strategy has been shown to yield an agreement of >95% for all enzyme digestions (6).

Patient interviews

Trained monitors conducted interviews with patients during hospital stay. Questions focused on past clinical history, occupation, and life-style. The respondent was the patient himself in 96% of the cases and a relative alone in 4% (1,4,33–35). Detailed occupational information was requested for ten activities *a priori* defined to be potentially related to pancreas and biliary cancers (2), including pesticide use, handling of petroleum derivatives, chemical industry, metal industry, rubber industry, graphic arts, jewellery, manufacture or repair of automobiles, leather tanning, and textile industry (33,34). Patients who reported having worked in any of these activities were asked for the duration of employment, specific activity, and products to which they had been exposed. The same information was also requested for two additional activities performed for at least 6 years. The occupations obtained were coded according to the Spanish National Classification of Occupations 1994, which is adapted from the International Standard Classification of Occupations 1988 (34).

Occupational exposure to organic solvents was assessed by two industrial hygienists (IH) and with the Finnish job-exposure matrix (Finjem). The IH assessed aliphatic hydrocarbon solvents, aromatic hydrocarbon solvents, benzene, chlorinated hydrocarbon solvents, and other organic solvents. They coded each subject as exposed, unexposed, or exposure unknown. 'Exposed' required a substantiated source of exposure. If exposure was unsubstantiated but possible, the category unknown was used. The intensity of the exposure was coded as high, low, none or unknown (33). The working definition for benzene exposure was 'any solvent with more than 1% of benzene'. Finjem assessed occupational exposure to aliphatic and alicyclic hydrocarbon solvents, aromatic hydrocarbon solvents, chlorinated hydrocarbon solvents, and other organic solvents (36). Two investigators (TK and JA) performed the conversion from Spanish to Finnish occupational codes (33). The exposure categories used were substantial, low, and unexposed. The cut point between low and substantial was set as close as possible to the 75 percentile of the distribution of the product of the probability of exposure (range 0.06–1) and the intensity of exposure (most in mg/m^3 or in p.p.m.) (33). For the present study we also created a more specific criterion to define exposure to solvents, by which exposure was required to have taken place based on both the IH and Finjem.

Statistical analyses

This case-case study (4) compared occupational exposures of EPC cases with and without K-*ras* mutated tumours. In contingency tables, comparison of two qualitative or categorical variables was performed with Pearson's χ^2 test for homogeneity or independence; alternatively, when ⩾20% of cells had expected counts less than five, Fisher's exact test was applied. For ordered categorical variables the Mantel–Haenszel χ^2 test for linear trend was used. If the observed number of cases in one cell of the contingency table was zero, the Woolf–Haldane correction was applied (37). Multivariate-adjusted odds ratios (OR) and their corresponding 95% confidence intervals (CI) were estimated by unconditional logistic regression. The following potential confounders were included in the models: sex, age, cumulative number of years smoked, and coffee consumption during the year prior to the first symptom. Allowance for other potential confounding variables (i.e. schooling, alcohol consumption, diabetes) did not substantially modify any of the estimates.

Results

The proportion of subjects who had been exposed to hydrocarbon solvents varied from 19% to 29% among K-*ras* mutated cases, depending on the type of exposure assessment (IH, Finjem or both); it varied from 4% to 25% among wild-type cases (Table I). An association between K-*ras* mutations and hydrocarbon solvent exposure was found with Finjem: mutated cases were over eight times more likely to have been occupationally exposed to hydrocarbon solvents than wild-type cases (OR = 8.93). With the IH assessment, the OR was 1.22 (0.38–4.21). The more specific criterion (exposure by IH and Finjem) yielded an estimate close to 6.

When looking separately at aliphatic and at aromatic hydrocarbon solvents, we observed that mutated cases were over six and seven times more likely, respectively, to have been exposed (Table I). No significant increases were seen using the IH evaluation. Overall, cases harbouring a K-*ras* mutation were twice as likely to have been exposed to benzene than wild-type cases. Among males the OR was 7.07 (95% CI: 1.01–145); only one woman was exposed to benzene.

While the IH did not classify any subject as exposed to chlorinated hydrocarbon solvents, results with Finjem suggest that in pancreatic cancer an association may exist between K-*ras* mutations and these compounds (Table I). The other organic solvents included alcohols, ketones, esters and glycol ethers. Their association with mutation status was similar to the ones seen for aliphatic and aromatic hydrocarbon solvents. The analysis by intensity of exposure revealed that there were no wild-type cases in the higher category in any of the five exposure groups assessed through Finjem.

In analyses restricted to subjects whose exposure to solvents started at least 10 years before diagnosis, the ORs were only slightly attenuated, and they all remained above 5; for instance, the OR for any hydrocarbon solvent was 7.01 (95% CI: 1.17–138).

A potential interaction was apparent between solvent exposure and smoking. Among non-smokers, all subjects exposed to solvents had mutated tumours. This was observed for all six exposure categories, and with both types of exposure assessment. Among smokers, by contrast, although the ORs were consistently elevated, some wild-type cases were exposed in each exposure category. Hence, the association between mutation and solvent exposure was weaker among smokers than among non-smokers. However, the interaction test for exposure to any hydrocarbon solvent was only close to statistical significance with IH ($P = 0.08$; $P = 0.45$ with Finjem), probably due to the small numbers.

The two most common K-*ras* mutations in EPC (1,5) were both strongly associated with solvent exposure, based on Finjem (Table II). Compared with K-*ras* wild-type cases, a significantly higher proportion of cases with a codon 12 mutation from glycine to valine (GGT⊖GTT) or to aspartic acid (GGT⊖GAT) were deemed exposed to a hydrocarbon solvent. This was not so with the IH assessment. Benzene maintained its two-fold OR among cases harbouring the two mentioned amino acid substitutions.

Table I. Occupational exposure to solvents among cases of exocrine pancreatic cancer with and without mutations in the K-*ras* gene

Exposure	K-*ras* mutated (n = 83)		K-*ras* wild-type (n = 24)		OR[a] (95% CI)
	Exposed no.	(%)	Exposed no.	(%)	
Any hydrocarbon solvent					
Industrial hygienists	24	(28.9)	6	(25.0)	1.22 (0.38–4.21)
Finjem	21	(25.3)	1	(4.2)	8.93 (1.55–172)
Both[b]	16	(19.3)	1	(4.2)	5.98 (0.98–117)
Aliphatic hydrocarbon solvents[c]					
Industrial hygienists	21	(25.3)	5	(20.8)	1.30 (0.37–5.02)
Finjem	16	(19.3)	1	(4.2)	6.55 (1.10–128)
Both	13	(15.7)	1	(4.2)	4.67 (0.74–93.3)
Aromatic hydrocarbon solvents					
Industrial hygienists	22	(26.5)	6	(25.0)	1.03 (0.30–3.73)
Finjem	18	(21.7)	1	(4.2)	7.57 (1.32–145)
Both	11	(13.3)	1	(4.2)	4.16 (0.63–85.0)
Benzene[d]					
Industrial hygienists	14	(16.9)	2	(8.3)	2.12 (0.46–15.3)
Chlorinated hydrocarbon solvents[e]					
Finjem	19	(22.9)	0	(0.0)	14.81[f](3.20–UH[g])
Other organic solvents					
Industrial hygienists	9	(10.8)	1	(4.2)	3.39 (0.54–67.1)
Finjem	19	(22.9)	1	(4.2)	8.36 (1.46–160)
Both	6	(7.2)	1	(4.2)	2.40 (0.34–50.0)

[a]Odds ratio adjusted by age, sex, and tobacco and coffee consumption.
[b]Exposed according to both industrial hygienists and Finjem.
[c]Aliphatic and alicyclic in Finjem.
[d]Exposure not assessed by Finjem.
[e]No subjects were deemed exposed by the industrial hygienists.
[f]Odds ratio based on the Woolf–Haldane correction.
[g]Unquantifiably high.

Table II. Organic solvents exposure based on Finjem and mutation spectra

	Valine vs K-*ras* wild-type[a]		Aspartic vs K-*ras* wild-type[a]	
	OR[b]	(95% CI)	OR[b]	(95% CI)
Any hydrocarbon solvent	11.04	(1.21–282)	17.45	(1.56–609)
Aliphatic and alicyclic hydrocarbon solvents	5.49	(0.37–161)	9.44	(0.62–370)
Aromatic hydrocarbon solvents	11.04	(1.21–282)	10.85	(0.85–410)
Chlorinated hydrocarbon solvents	12.56[c]	(2.57–UH[d])	23.58[c]	(5.47–UH[d])
Other organic solvents	7.14	(0.62–197)	14.48	(1.14–549)

[a]Number of cases mutated from glycine (GGT) to valine (GTT): 21; mutated to aspartic acid (GAT): 19.
[b]Odds ratio adjusted by age, sex, and tobacco and coffee consumption.
[c]Odds ratio based on the Woolf–Haldane correction.
[d]Unquantifiably high.

Discussion

Results obtained with Finjem and with the combination of Finjem and IH (but not with IH alone) support the hypothesis of an association between occupational exposure to hydrocarbon solvents and mutational activation of the K-*ras* gene in exocrine pancreatic cancer. Occupational exposure to organic solvents, particularly to chlorinated solvents, may be a risk factor for pancreatic cancer (2). In animal models 1,3-butadiene and vinyl chloride, a degradation product of chloroethylene solvents (perchloroethylene and trichloroethylene), may induce *ras* mutated tumours (8,38). In humans, occupational exposure to hydrocarbon solvents might be associated with *ras* gene activation in acute myeloid leukaemia (12). Vinyl chloride has putatively been linked to *ras* alterations in humans (18,22,27). In contrast, several studies (all based in <50 subjects) have failed to correlate exposure to organic solvents, mainly benzene, and altered expression of *ras* genes in healthy human populations (15–17).

Genes of the *ras* family are mutated in human malignant and pre-malignant lesions in several anatomic locations, including the endometrium, thyroid, colon, gallbladder and biliary tract, lung, and in leukemias. Several other lines of evidence (1,3–5,39,40) suggest that *ras* activation may be an early event in the carcinogenic process. In pancreas cancer, too, K-*ras* is found activated in pre-malignant pancreatic lesions (5,40). Studies on the frequency and spectra of *ras* mutations support the notion that its activation and expression may be chemically related, rather than spontaneous (1,4,39–44).

Human molecular studies on the mutational spectra of *ras* have a sound biological basis: *ras* genes are highly conserved in vertebrates; they are mainly activated by point mutations; are found activated in many human tumours; and the limited

number of codons at which activating mutations occur makes feasible their study in large numbers of people (3,39,41). The mutation spectrum is the result of different processes, including mutagen activation, DNA damage, lack of repair, polymerase misreading and biological selection. Hence, the findings reported here may fit with at least three broad mechanistic scenarios: enzymatic induction of K-*ras* mutagens; direct K-*ras* damage; and provision of a proliferation advantage to K-*ras* mutated cell clones. If confirmed by new studies, the similar association of solvent exposure with the two main mutations (Table II) might preferentially support indirect mechanisms such as those operating in the first and third scenarios.

In humans, occupational exposures to different types of hydrocarbon solvents are highly correlated; thus, complex studies are needed to assess the specific effects of each agent and to control residual confounding. Nonetheless, current knowledge on carcinogenic effects of most hydrocarbon solvents is also in accordance with the lack of a direct mutagenic effect. White spirit (a typical mixture of primarily aliphatic hydrocarbons) does not test positive for mutagenicity (45). Hexane has proved to be genotoxic in mammals, but not mutagenic (30). Among aromatic solvents, benzene can induce GC > AT transitions and GC > TA transversions (46); it is weakly effective in inducing point mutations, mainly inducing deletions and large-scale chromosomal alterations (47). Styrene exposed workers showed higher levels of O^6-styrene guanine adducts and an elevated rate of hypoxanthine guanine phosphoribosyl transferase (HPRT) mutant frequency (48). O^6-guanine adducts are considered pro-mutagenic lesions that may give rise to GC > AT transitions (49). Other aromatic solvents as xylenes or toluene have not shown mutagenic properties (31). With respect to chlorinated solvents, the mutation profile observed for the H-*ras* gene in liver tumours induced by methylene chloride is similar to that seen in spontaneous liver tumours, hence suggesting that methylene chloride acts in the liver by promoting cells with spontaneous lesions (50). Similar findings have been reported for K-*ras* mutations in lung cancer, suggesting that methylene chloride may cause early and persistent loss of growth control in lung cells (51). The possibility of enzymatic induction of K-*ras* mutagens is illustrated by the fact that organic solvents are the substrate of several polymorphic enzymes (52,53) that may be associated with *ras* mutations (54).

One strength of this study is the high percentage of subjects (90%) with occupational information personally obtained around the time of diagnosis (33,34). Other epidemiologic studies on pancreatic cancer achieved response rates of 40–60%. The proportion of cases with both molecular and environmental data (58%) is also high for EPC (6). Lack of differences between patients included and excluded argues against an important selection bias (4). The observed prevalence of K-*ras* mutations (78%) agrees with that found by the larger studies (5,6). Since K-*ras* status and occupational exposures were assessed independently, misclassification of exposure would be non-differential.

In our previous case–control analyses, the combined group of cases (mutated and wild-type) did not have significantly greater exposure to organic solvents than controls (33). Yet, it has been shown that this is compatible with a higher exposure among mutated cases (7,55). Since our previous controls included some patients with disorders where *ras* alterations can exist, we chose not to use them in the present study.

Experts' assessment of occupational exposures and job-exposure matrixes (JEMs) have both strengths and weaknesses. The IH were familiar with the country working conditions, and took advantage of information about gender, periods in which cases were employed, and products to which patients referred being exposed (used in up to 40% of cases). However, in most cases the information provided by participants was just a job title. The sensitivity of a JEM for occupational exposures to solvents may be lower than that of experts' assessment when using limited occupational information, but there are also concerns about the validity of experts' judgements, since they inevitably have a subjective component (56,57). When a JEM involves specific elements such as level, probability and time period of exposure, risk estimates are more accurate (58). The most frequent occupations among cases identified as exposed to solvents by both approaches include lasters and sole fitters, machine and engine mechanics, motor vehicle and tram drivers, and painters. Glues, varnishes and cleaning solvents were the main sources of exposure to hydrocarbon solvents. The two assessment methods showed a high correlation between exposure to aromatic and to aliphatic hydrocarbon solvents, to the extent that it was hardly possible to distinguish whether the associations with K-*ras* were due to the first type of solvents or to the latter. The main source of discrepancy between Finjem and IH—and subsequently the main reason for their partly discrepant results—was that IH deemed as substantial the information on exposure to solvents among farmers that applied pesticides ($n = 7$), while Finjem does not consider this information. In Spain, pesticide application was an occasional activity (about once per year) rather than a frequent task among farmers working between 1950 and 1980, and the cumulative exposure to solvents in these cases is likely to be quite different from that experienced by workers in other occupations like lasters, engine mechanics or painters.

Ras activation has been proposed as a potential biomarker of occupational cancer risk that might allow early detection (19). However, before implementing systematic screening for *ras* alterations in the workplace, large follow-up studies would need to evaluate the prognostic significance of *ras* alterations in asymptomatic subjects. Furthermore, this is only the first study on occupational exposures and K-*ras* activation in pancreatic cancer. While it stems from the largest study with environmental and molecular information in pancreatic cancer, some estimates were imprecise, discrepant results were obtained with IH and Finjem, and results may not apply to other populations. Therefore, studies able to refute or to confirm our findings are required before policy implications, if any, are assessed.

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